Keratinocytes Synthesize Enteropeptidase and Multiple Forms of Trypsinogen during Terminal Differentiation

Jotaro Nakanishi[1], Mami Yamamoto[1,2], Junichi Koyama[1], Junko Sato[1] and Toshihiko Hibino[1]

Members of the trypsin-like and chymotrypsin-like kallikrein family are important in the desquamation process. In this study, we isolated cDNA clones encoding trypsinogen 4 (brain trypsinogen) and a previously unreported isoform of trypsinogen from a human keratinocyte cDNA library. The nucleotide sequence of the new isoform only differs from those of trypsinogen 3 (mesotrypsinogen) and trypsinogen 4 in an exon encoding the N-terminal region, indicating that this isoform is an alternative splicing variant of the mesotrypsinogen gene *PRSS3*. Both isoforms contained the sequence DDDDK-I, a putative cleavage site for activation by enteropeptidase. Thus, after activation, mesotrypsin would be produced. Immunohistochemical and *in situ* hybridization studies revealed that trypsinogens were expressed and localized in the upper epidermis, especially in the granular layer. In cultured keratinocytes, enteropeptidase mRNA was expressed at the confluent stage, and its expression was strongly upregulated after air exposure. Interestingly, it was synthesized and localized only at the granular layer, suggesting that the generation of active mesotrypsin is restricted to this layer. The enteropeptidase-cleavage product was also found at the same layer. When a skin equivalent model was cultured in the medium without air exposure, the cornified layer was not formed, and many cells expressed trypsinogens and enteropeptidase. Those cells were found to be TUNEL positive. Because mesotrypsin is resistant to naturally occurring trypsin inhibitors, confined expression of the isoforms of mesotrypsinogens and enteropeptidase may indicate that mesotrypsin is involved in keratinocyte terminal differentiation.

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INTRODUCTION

Stratum corneum (SC), the outermost layer of epidermis, consists of cornified cells (corneocytes) and continuous extracellular lipid layers, forming a physicochemical barrier that efficiently protects the body from environmental hazards. The SC is continuously produced through the terminal differentiation of keratinocytes. At the same time, cell shedding (desquamation) also occurs to maintain a constant thickness of the SC. In normal desquamation, individual corneocytes or small aggregates become detached from the skin surface. The process is imperceptible to healthy individuals, but in some pathological conditions there is an imbalance between SC production and desquamation, which results in excessive visible scales. This is seen in inflammatory skin diseases such as psoriasis (Fartasch, 1997; Langley *et al.*, 2005).

Lundstrom and Egelrud reported that a chymotrypsin-like serine protease is present in the SC and that it is involved in desquamation (Lundstrom and Egelrud, 1990; Egelrud and Lundstrom, 1991). This enzyme was purified as stratum corneum chymotryptic enzyme from human plantar SC, and cDNA encoding stratum corneum chymotryptic enzyme was isolated from a human keratinocyte cDNA library (Egelrud, 1993; Hansson *et al.*, 1994). We have shown that not only a chymotryptic enzyme, but also trypsin-like serine protease(s) is involved in the desquamation process of nonplantar SC (Suzuki *et al.*, 1993, 1994, 1996). Recent studies have revealed that a human tissue kallikrein gene family encoding 15 serine proteases, designated kallikrein 1-15 (KLK1-15), is located as a cluster on chromosome 19q13.4 (Harvey *et al.*, 2000; Clements *et al.*, 2001). Many of these serine proteases are widely expressed in human tissues (Harvey *et al.*, 2000), including skin (Komatsu *et al.*, 2005). The proteases previously identified as being involved in desquamation were then renamed as kallikrein family members: stratum corneum chymotryptic enzyme was renamed kallikrein 7 (KLK7, hK7) (Yousef *et al.*, 2000) and trypsin-like enzyme (Brattsand and Egelrud, 1999; Ekholm *et al.*, 2000; Caubet

[1]*Shiseido Research Center, Yokohama, Japan and* [2]*Department of Dermatology, Tokyo Medical University, Nishishinjuku, Shinjuku-ku, Tokyo, Japan*

Correspondence: Toshihiko Hibino, Shiseido Research Center, 2-12-1 Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan.
E-mail: toshihiko.hibino@to.shiseido.co.jp

Abbreviations: DIG, digoxigenin; ECS, enteropeptidase cleavage site; hK, tissue kallikrein protein; KLK, tissue kallikrein gene; SC, stratum corneum

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et al., 2004) was renamed KLK5 (hK5). There is much evidence that KLK5 and KLK7 have critical functions in desquamation. Furthermore, Komatsu *et al.* (2005) showed that KLK6, KLK8, KLK10, KLK11, KLK13 and KLK14 are also present in the SC, and suggested that these enzymes might also be involved in desquamation, acting as a proteolytic cascade. This would be consistent with the finding that KLK5 activates KLK7 and KLK14 at weakly acidic pH (Brattsand *et al.*, 2005).

On the other hand, three different trypsinogens were present in human pancreatic juice. On the basis of their isoelectric points, they are called cationic trypsinogen, anionic trypsinogen and mesotrypsinogen. Recent studies revealed that these trypsinogens are encoded by distinct genes, *PRSS1*, *PRSS2* and *PRSS3*, respectively (Sahin-Toth, 2005). The *PRSS1* gene product, trypsinogen 1 and the *PRSS2* gene product, trypsinogen 2 are major digestive pancreatic proteases. The *PRSS3* is the mesotrypsinogen gene and at least two splicing variants would be produced (trypsinogens 3 and 4). All of these trypsinogens possess the putative cleavage site sequence DDDDK-I for the activation by enteropeptidase (Light and Janska, 1989). Trypsinogens are expressed not only in the pancreas, but also in other tissues, including epithelial cells of various tissues (Koshikawa *et al.*, 1997) and human brain (Wiegand *et al.*, 1993). Interestingly, mesotrypsin differs from other two trypsins in its substrate specificity and inhibitor susceptibility. Mesotrypsin cleaves protein substrates poorly. The most characteristic feature is the resistance to the naturally occurring trypsin inhibitors, such as α1-trypsin inhibitor, pancreatic secretory trypsin inhibitor (Kazal type), soybean trypsin inhibitor (Kunitz type) (Rinderknecht *et al.*, 1984; Nyaruhucha *et al.*, 1997). These findings indicate that trypsin could be involved in various physiological reactions, in addition to intestinal digestion.

Here we report the cDNA cloning of trypsinogen genes from a human keratinocyte cDNA library. We isolated two splicing isoforms of mesotrypsinogen gene *PRSS3*, of which one is identical with trypsinogen 4 (brain trypsinogen) and the other is a previously unknown isoform, differing only in the exon encoding the N-terminal region. Both isoforms contain the activation sequence DDDDK-I, and we found that the activating enteropeptidase was localized exclusively in the granular layer of the epidermis. The characteristic expression and localization of these trypsinogens and the activating enzyme are consistent with the idea that mesotrypsin is involved in keratinocyte terminal differentiation.

RESULTS

Molecular cloning of trypsinogens from a human keratinocyte cDNA library

Reverse transcriptase (RT)–PCR amplification using a degenerate primer pair (see Materials and Methods section) yielded 540-bp PCR products, which were subcloned into the pCRII vector. The sequences of all clones tested were identical with that of trypsinogen 3 or 4, and one of the clones (Tryp 1) was used as a probe for screening of a human keratinocyte cDNA library in the λgt11 vector. Among 50 positive clones, two contained an open reading frame of 912 nucleotides, which encodes 304 amino acids. The encoded product was identified as trypsinogen 4 (brain trypsinogen) (data not shown; DDBJ/EMBL/GenBank accession number AB298285). Of these 50 positive clones, 46 were also categorized as trypsinogen 4 by sequence analysis, although they did not contain the full-length open reading frame. The remaining two clones had an open reading frame of 720 nucleotides, which encodes a 240-amino-acid isoform of trypsinogen. To our knowledge, this is previously unreported and registered at DDBJ/EMBL/GenBank (accession number AB298286; Figure 1).

Comparison of the deduced amino-acid sequence of the new isoform with known trypsinogen sequences

The nucleotide sequence and the deduced amino-acid sequence of the new isoform differ from those of trypsinogens 3 and 4 in the sequence at the N-terminal region (Figure 2). The deduced amino-acid sequence of the N-terminal region for this isoform, MGPAGEVAV, is the shortest among the trypsinogens, and we named this new isoform trypsinogen 5. This enzyme contains the putative activation cleavage site (DDDDK-I), and after cleavage, this isoform, as well as trypsinogen 4, generates an enzyme identical with mesotrypsin. A major characteristic feature of mesotrypsin is arginine at position 191 of trypsinogen 5, in proximity to the active serine at position 193. It has been reported that this single mutation from glycine of other trypsins (cationic and anionic trypsins) to arginine in mesotrypsin is responsible for the lack of susceptibility of mesotrypsin to natural trypsin inhibitors (Szmola *et al.*, 2003).

Genomic organization of epidermal trypsinogens

The genomic organization of the exons encoding the N-terminal region of trypsinogens 3, 4 and 5 is shown in Figure 3. These isoforms use three different exons for the ATG start codon. The exon encoding the N terminus of the new isoform (trypsinogen 5) was found just behind the exon encoding the N terminus of trypsinogen 4 on the reported human genomic DNA sequence (Wiegand *et al.*, 1993). These segments are located at 45-kb upstream of the exon used for trypsinogen 3, indicating that these trypsinogens are splicing variants of the *PRSS3* gene.

Tissue distribution and expression of trypsinogens 4 and 5

The expression of trypsinogens 4 and 5 mRNAs in various human tissues was examined by RT–PCR. As shown in Figure 4a, trypsinogen 4 mRNA was expressed in all tissues and cells tested, brain, heart, lung, liver, stomach, small intestine, pancreas, spleen, kidney, prostate, testis, uterus, placenta, skin fibroblasts and keratinocytes, except skeletal muscle. In contrast, expression of trypsinogen 5 was restricted to brain, stomach, small intestine, uterus, fibroblasts and keratinocytes. The expression of pancreatic trypsinogens (trypsinogens 1, 2 and 3) was observed only in pancreas and lung (data not shown). We also performed northern blot analysis for the identification of trypsinogen mRNAs. In cultured keratinocytes, only one trypsinogen mRNA (probably trypsinogen 4) was shown (Figure 4b). Because trypsinogen 5 was

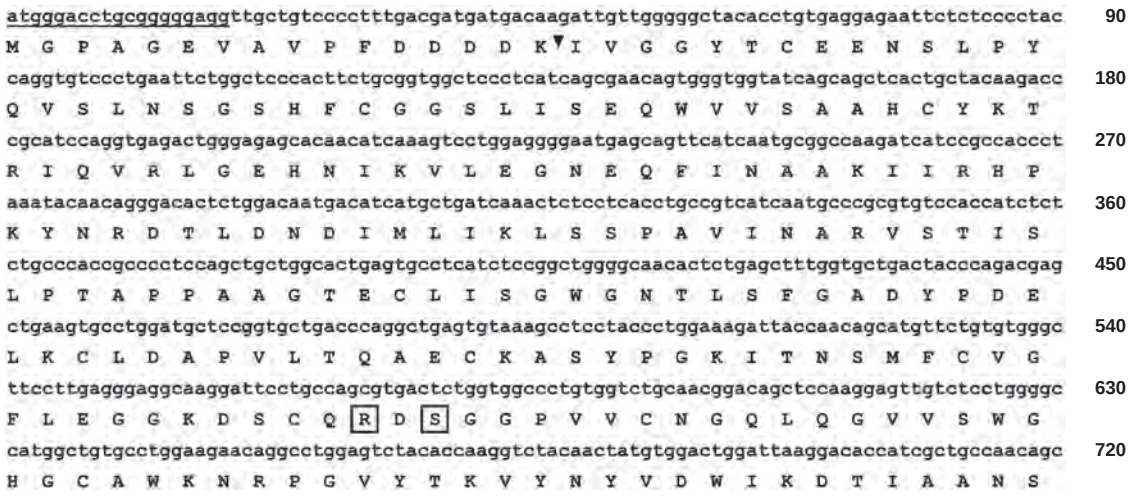

Figure 1. The nucleotide and deduced amino-acid sequences of the new isoform of trypsinogen. The nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank databases with the accession number AB298286. The unique sequence encoding the N-terminal region is indicated with an underline. One-letter codes for amino acids are shown in the lower line. An arrowhead indicates the enteropeptidase activation site. Active site serine (S^{193}) and a critical amino acid responsible for inhibitor resistance (R^{191}) are boxed.

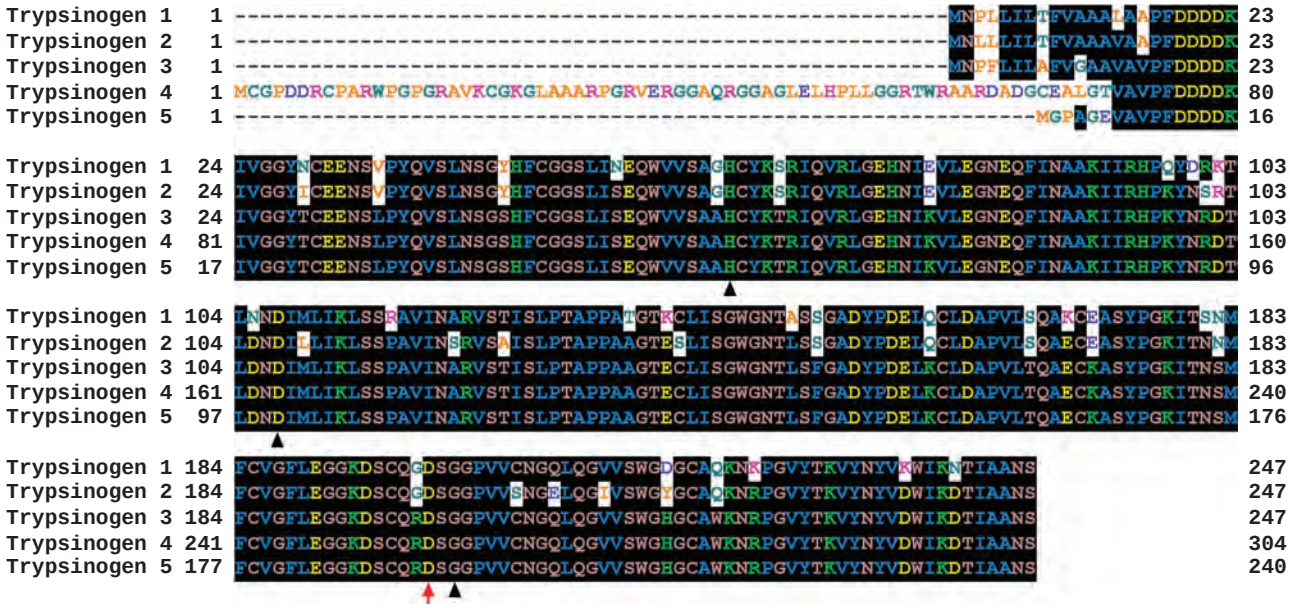

Figure 2. Comparison of the deduced amino-acid sequences of human trypsinogens. Trypsinogens 1 (cationic trypsinogen) and 2 (anionic trypsinogen) are encoded by the *PRSS1* and *PRSS2* genes, respectively. Trypsinogens 3 (mesotrypsinogen), 4 (brain trypsinogen) and 5 (new isoform) are encoded by the *PRSS3* gene. Those amino acids that are different among five trypsinogens are shown with a white background. Arrowheads show the conserved catalytic triad of amino acids. An arrow indicates a unique amino acid arginine (R) that characterizes inhibitor resistance in mesotrypsin.

supposed to be a minor species compared with trypsinogen 4 as shown in the cloning process (2 clones vs 48 clones), we assume that our current method for northern blot would not have enough sensitivity to detect trypsinogen 5.

Western blot analysis showed the presence of 34 and 26 kDa bands in the extracts from proliferating keratinocytes (80% confluent) as well as differentiated keratinocytes (confluent and air exposed; Figure 4c). These bands are likely to correspond to trypsinogens 4 and 5 from the deduced molecular weights of 32,498 and 25,807 Da, respectively. In addition, a 24 kDa band was detected only in the extract from the differentiated keratinocytes, indicating the presence of active mesotrypsin in this preparation. In addition, we analyzed the presence of trypsinogens and trypsins in various tissue extracts by western blot (Figure 4d). We found that the major form of trypsinogen was detected near 34 kDa, which was predominant in pancreas, followed by lung, liver and kidney. Only a weak band with 34 kDa was

Figure 3. Genomic organization of the exons encoding the N-terminal region of three isoforms of *PRSS3* gene. The coding sequences including ATG start codon of three isoforms are given in capital letters. The exon–intron junctions are underlined, although the 5′-ends of these exons are undetermined.

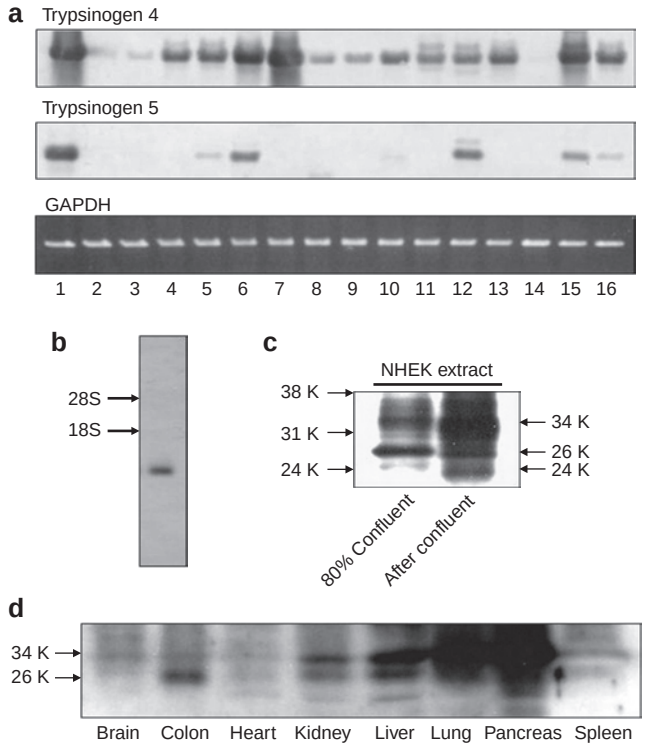

Figure 4. Expression of trypsinogen isoforms in skin and various tissues. (**a**) mRNA expression of trypsinogens 4 and 5 in various tissues. Reverse transcriptase (RT)–PCR products were detected by Southern blotting as described in the Materials and Methods section. Lane 1, brain; 2, heart; 3, lung; 4, liver; 5, stomach; 6, small intestine; 7, pancreas; 8, spleen; 9, kidney; 10, prostate; 11, testis; 12, uterus; 13, placenta; 14, skeletal muscle; 15, keratinocytes; 16, fibroblasts. (**b**) Northern blot analysis of epidermal trypsinogen in cultured human keratinocytes. Only a single species (trypsinogen 4) can be detected probably due to the abundance of this trypsinogen. (**c**) Western blot analysis of trypsinogens and trypsins in keratinocytes. Extracts obtained from proliferating keratinocytes and prolonged culture after differentiation were examined. (**d**) Western blot analysis of trypsinogens and trypsins in various tissue extracts.

found for brain. In heart, trypsinogen was hardly detectable. Relatively abundant low molecular weight species with 26 kDa was shown for colon and liver.

Expression and localization of trypsinogens in human skin

We examined the expression and localization of trypsinogen in human skin. First, we performed *in situ* hybridization using a digoxigenin (DIG)-labeled cRNA probe that recognizes all trypsinogen mRNAs (Figure 5a). In the epidermis, trypsinogen mRNAs were expressed in suprabasal cells, especially in granular cells. On the other hand, no expression was observed in basal cells. An immunohistochemical study using anti-trypsin antibody showed a rather confined localization of trypsin(ogen)s in the upper epidermis, mostly in granular cells (Figure 5b). Nonimmune normal rabbit IgG was used as a negative control and did not show any positive staining except a weak staining at the edge of the epidermis, which indicates nonspecific staining (Figure 5c).

Expression of enteropeptidase in human keratinocytes and epidermis

Because epidermal trypsinogens contain the enteropeptidase activation sequence (DDDDK-I), we investigated the expression of enteropeptidase mRNA in cultured keratinocytes (Figure 6a). RT–PCR showed that enteropeptidase was expressed at the confluent stage, and was unaffected by a high calcium concentration. In contrast, when keratinocytes were exposed to air and further incubated in the presence of high calcium concentration, the expression of enteropeptidase was strongly upregulated. *In situ* hybridization study showed that enteropeptidase mRNA was detected only in those cells that aggregated and piled up after strong differentiation stimuli (confluent and air exposed; Figure 6b). In the human skin, the enteropeptidase mRNA was detected exclusively in the granular layer (Figure 6c). Interestingly, the staining was confined to a very limited number of granular cells.

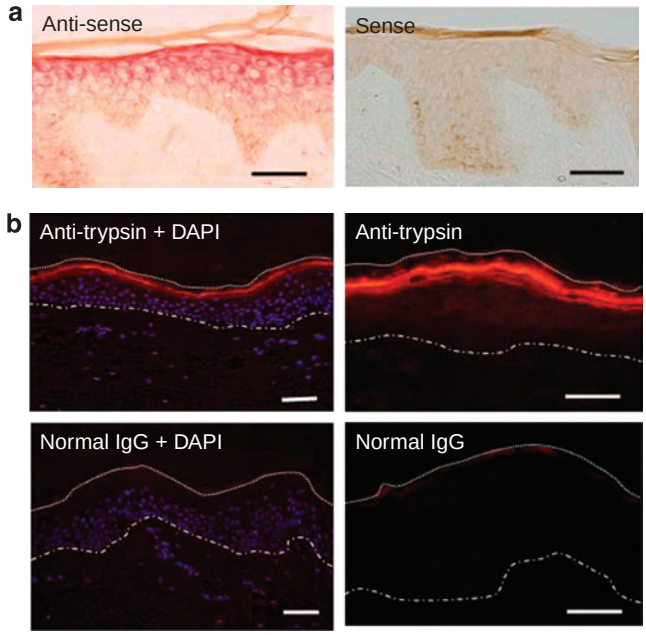

Figure 5. Expression and localization of trypsinogen in human skin. (**a**) *In situ* hybridization of trypsinogen in human epidermis. Anti-sense (left) and sense (right) cRNA probes that recognize all trypsinogens were prepared as described in the Materials and Methods section. Color was developed with Vector Red. (**b**) Immunohistochemical localization of trypsinogen. Rabbit anti-human trypsin IgG that recognizes all trypsin isoforms was used as a primary antibody. Fluorescence detection was performed using Alexa Fluor 555 and nuclear staining was carried out with 4′,6-diamino-2-phenylindole. Lower (left) and higher (right) magnifications are shown. Nonimmune normal rabbit IgG was used as a negative control. Outermost cornified layer is marked with a dotted white line. Dermo-epidermal junction is delineated with a broken white line. Bar = 50 μm.

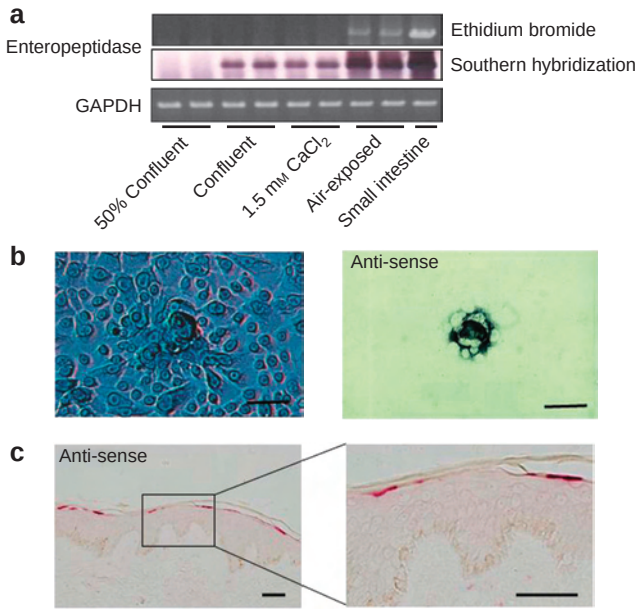

Figure 6. Expression of enteropeptidase in human keratinocytes and skin. (**a**) mRNA expression of enteropeptidase in human keratinocytes at various stages of culture condition. Reverse transcriptase (RT)–PCR products were visualized with ethidium bromide (upper panel) and analyzed with Southern blotting by means of the enteropeptidase-specific cDNA probe. (**b**) *In situ* hybridization of enteropeptidase in cultured human keratinocytes. Keratinocytes were cultured until the confluent monolayer. After removing the medium, keratinocytes were kept for 15 minutes in the air with lid and further incubated for 2 days in the serum-free medium including 2 mM Ca^{2+} (left). *In situ* hybridization of enteropeptidase was performed as described in the Materials and Methods section (right). Bar = 20 μm. (**c**) *In situ* hybridization of enteropeptidase in human epidermis. Color images are developed with Vector Red. Lower (left) and higher (right) magnifications are shown. Bar = 100 μm.

Localization of enteropeptidase and its cleavage product in human skin

Immunohistochemical study showed that enteropeptidase was localized also in the very confined area of granular cells (Figure 7a). This was coincided with the results of *in situ* hybridization. Furthermore, staining with an antibody directed to the enteropeptidase cleavage site (ECS) (DDDDK-I) revealed that the cleavage product was present similarly at the granular layer (Figure 7b). These results suggest that the conversion of trypsinogen to trypsin was occurring at the very restricted site of the granular layer.

Analyses with skin equivalent models

We performed limited attempts to elucidate physiological functions of enteropeptidase–trypsin system using skin equivalent models (Figure 8). For this purpose, we prepared two kinds of models: one is normally lifted to the air–liquid interface (air exposed; Figure 8, left panels) and the other was immersed in the medium throughout the experiments (dipped; right panels). Immunostaining of trypsin(ogen)s showed broader localization from basal to granular cells (Figure 8a, left) compared with the healthy human skin. In contrast, morphological difference was evident in the dipped

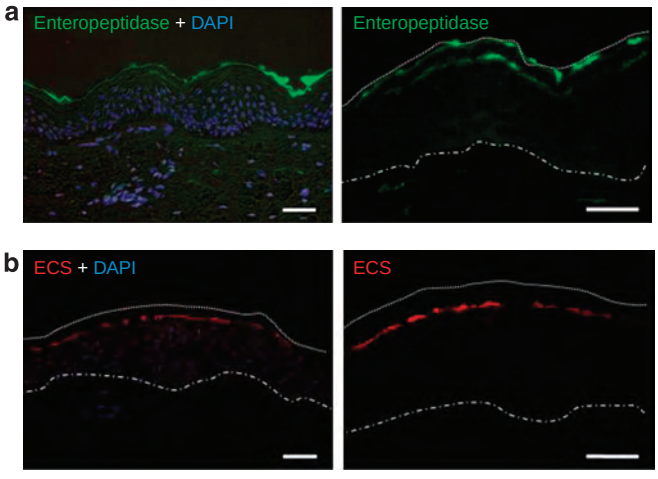

Figure 7. Localization of enteropeptidase and its cleavage product in human epidermis. (**a**) Enteropeptidase is present in the granular layer (left). An enlarged figure is also shown (right). Staining of the skin surface was nonspecific. (**b**) The enteropeptidase-cleavage product is present similarly in the restricted area of granular cells (left). An enlarged figure is also shown (right). Outermost cornified layer is marked with a dotted white line. Dermo-epidermal junction is delineated with a broken white line. Bar = 100 μm.

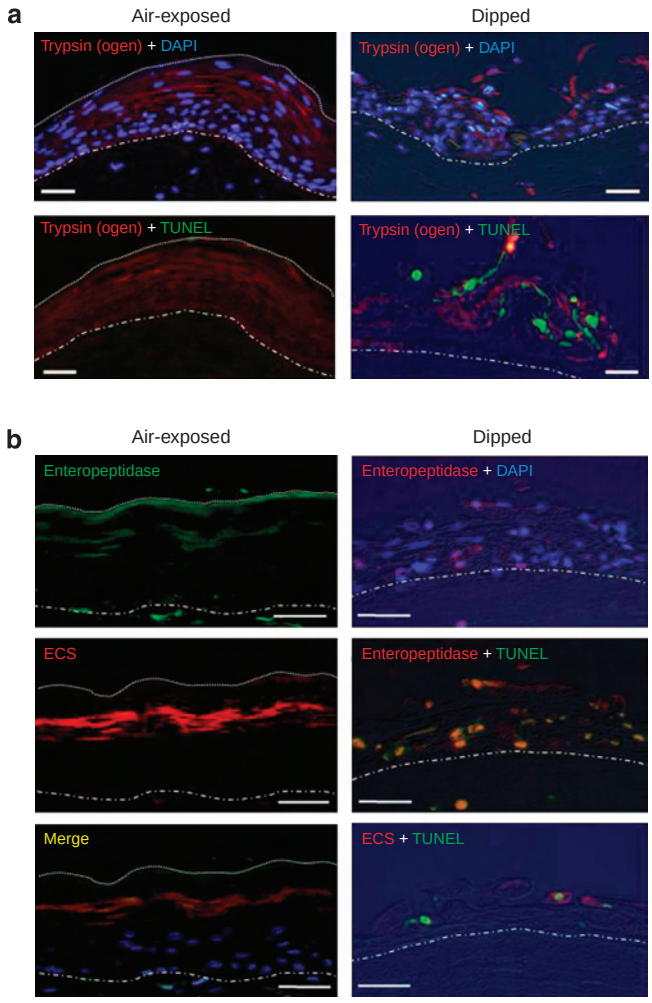

Figure 8. Analyses with skin equivalent models. Two different skin equivalent models were prepared. One is normally developed by lifting the model to an air–liquid interface (left panels). The other is kept in the medium throughout the experiment (right panels). (**a**) Immunostaining for trypsin(ogen)s with nuclei staining is shown in the upper panel. Lower panel shows double staining for trypsin(ogen)s and TdT-mediated dUTP-biotin nick end labeling (TUNEL). Many TUNEL-positive cells are observed only in the dipped model (lower right). (**b**) Immunostaining of enteropeptidase and enteropeptidase cleavage site (ECS) with a merged figure is shown for the air-exposed culture (left panels). Enteropeptidase with nuclear staining and double stainings for enteropeptidase + TUNEL and ECS + TUNEL are shown in the dipped culture model (right panels). Outermost cornified layer is marked with a dotted white line. Dermo-epidermal junction is delineated with a broken white line. Bar = 100 μm.

culture model (Figure 8a, right). The cornified layer was not formed and stratified structure was no more observed. Trypsin(ogen)s were expressed strongly in some particular cells and many of these cells were TUNEL positive. In the air-exposed culture, TUNEL-positive cells could not be found (Figure 8b, left). In the dipped culture, continuous staining was not observed and enteropeptidase-positive cells showed scattered distribution (Figure 8b, right). TUNEL staining showed that most of these cells were dying by apoptosis. ECS was detected similarly and these cells were also TUNEL-positive.

DISCUSSION

Previous studies have shown that trypsin-like and chymotrypsin-like serine proteases are present in human SC and are involved in the process of desquamation (Lundstrom and Egelrud, 1990; Egelrud and Lundstrom, 1991; Suzuki *et al.*, 1993, 1994, 1996). These enzymes are currently classified as members of the kallikrein family. The activities of the epidermal kallikreins toward corneodesmosome proteins have been well characterized (Caubet *et al.*, 2004), and the existence of a proteolytic cascade of kallikreins in the SC has been reported (Brattsand *et al.*, 2005). On the other hand, it has not been clear whether trypsin(ogen) itself is expressed in the skin.

In this study, we have cloned two kinds of trypsinogen cDNAs, which encode trypsinogen 4 (brain trypsinogen) and its new alternative splicing variant, from a human keratinocyte cDNA library. We named the new isoform trypsinogen 5. The trypsinogen 4 gene has already been cloned from human brain by means of the RACE (rapid amplification of cDNA ends) technique, but it did not contain an ATG-start codon (Wiegand *et al*, 1993), probably because the GC-rich sequence in the 5′-region caused incomplete reverse transcription or PCR amplification. Here, we succeeded in obtaining the complete sequence including the ATG-start codon. As shown in Figure 3, it appears that trypsinogens 3 (mesotrypsinogen), 4 (brain trypsinogen) and 5 (new isoform) are derived from the same gene, *PRSS3*. Interestingly, the active forms of these isoforms are identical with mesotrypsin, indicating that the differences in the N-terminal regions are associated with the tissue-specific expression of these trypsinogens. Indeed, mRNA expression of trypsinogen 5 was quite restricted and only found in brain, stomach, small intestine, uterus, fibroblasts and keratinocytes. In contrast, trypsinogen 4 was detected in all the tissues except skeletal muscle tested in this study (Figure 4a). Western blot analysis showed that trypsinogens were expressed in many tissues except heart, although their expression levels varied from tissue to tissue (Figure 4d). Thus, it is quite likely that trypsins are necessary not only for digestive processes but also for comprehensive physiological reactions in many tissues.

According to the deduced amino-acid sequence (Figure 2), trypsinogens 4 and 5 lack a signal peptide sequence, unlike trypsinogens 1, 2 and 3, suggesting an intracellular function. However, it remains possible that epidermal trypsin(ogen)s may be secreted into the extracellular space by lamellar bodies. In the transition between the stratum granulosum and the SC, the lamellar bodies fuse with the plasma membrane and secrete their contents to the SC extracellular space. Kallikreins are transported to the extracellular space by lamellar bodies (Menon *et al.*, 1992; Sondell *et al.*, 1995). The same system may be used for trypsin(ogen)s.

Trypsinogens 4 and 5 both contain the activation cleavage site (DDDDK-I), which is a highly conserved sequence found in pancreatic trypsinogens from various species (Bricteux-Gregoire *et al.*, 1971). The sequence DDDDK-I is poorly cleaved by trypsin itself, because of the predominance of negatively charged residues at the cleavage site (Abita *et al.*,

1969). In contrast, enteropeptidase has high specificity for the DDDDK-I sequence (Light and Janska, 1989). For example, the catalytic efficiency of bovine enteropeptidase is 34,000-fold greater than that of bovine trypsin toward bovine trypsinogen (Anderson *et al.*, 1977). Therefore, enteropeptidase is the only enzyme capable of physiologically converting trypsinogen to trypsin. The expression of enteropeptidase has been reported to be abundant in small intestine and duodenal mucosa of various species, but there is no information about the expression in other tissues, except for one report of weak mRNA expression in stomach, colon and brain of rat (Yahagi *et al.*, 1996).

In this study, we have detected strong expression of enteropeptidase mRNA and the protein exclusively in the granular layer of human epidermis (Figures 6c and 7a). The expression of this enzyme may be associated with the final stage of the terminal differentiation; indeed significant upregulation was observed only when the monolayer culture was exposed to air after confluency (Figure 6a). On the other hand, trypsinogen was expressed from the suprabasal layer to the granular layer, and heavy immunostaining for the protein was observed at the granular layer (Figure 5), indicating that the activation of epidermal trypsinogens is tightly regulated spatiotemporally and is dependent on enteropeptidase. Indeed by use of an antibody directed to the ECS (DDDDK-I), we confirmed that its cleavage product was present similarly at the granular layer of the human epidermis. Because of its unique sequence, it is highly likely that mesotypsin is generated at the same site.

During terminal differentiation, many events are associated with proteolytic action. In the uppermost granular cells, the nuclei and all organelles disappear, the molecular weights of keratins decrease (Fuchs and Green, 1980; Bowden *et al.*, 1984) and profilaggrin is processed to filaggrin (Fleckman *et al.*, 1985). Recent investigations have shown the importance of the serine protease inhibitor, lympho-epithelial-Kazal-type-5 inhibitor (LEKTI) in desquamation. Mutation of LEKTI gene, *SPINK5*, was identified as a defective gene in patients with a severe autosomal recessive ichthyosiform skin condition known as Netherton syndrome (Chavanas *et al.*, 2000). The major desquamation proteases KLK5 and KLK7 are strongly inhibited by LEKTI (Schechter *et al.*, 2005). SPINK5-deficient mice show hyperactivity of epidermal proteases and mimic Netherton syndrome through excessive degradation of desmoglein 1 (Descargues *et al.*, 2005). Multiple epidermal kallikreins may also participate in desquamation through cleavage of desmoglein 1, and they are regulated by LEKTI (Borgono *et al.*, 2007).

Apparently, these events are regulated by the balance between proteases and protease inhibitors. Because epidermal trypsins are identical with mesotrypsin, the active enzymes are inhibitor resistant (Nyaruhucha *et al.*, 1997). Furthermore, mesotrypsin is a unique digestive protease for naturally occurring trypsin inhibitors (Szmola *et al.*, 2003; Sahin-Toth, 2005). Thus, once mesotrypsin is generated, it will change the balance between kallikreins and LEKTI. It is tempting to speculate that overactivation of mesotrypsin has similar effect with loss of LEKTI, causing proteolysis of the cornified layer (Hachem *et al.*, 2006). Using skin equivalent models, we made a limited attempt to find a clue to physiological functions of mesotrypsin in the epidermis. In the dipped culture model, we showed that cornified layer could not be formed because of the apoptosis of keratinocyte. Interestingly, those keratinocytes showed colocalization of trypsin(ogens), enteropeptidase and its cleavage product with TUNEL positivity (Figure 8). Our findings suggest that epidermal mesotrypsin may have a critical function in terminal differentiation and desquamation. Deregulation of this enzyme could be a critical event that would cause abnormal differentiation and disorders of the skin.

MATERIALS AND METHODS

Cell culture

Human keratinocytes derived from normal foreskin (Cascade Biologics, Portland, ME) were cultured in keratinocyte growth medium composed of MCDB 153 medium supplemented with epidermal growth factor (0.1 ng ml^{-1}), insulin (10 μg ml^{-1}), hydrocortisone (0.5 μg ml^{-1}), bovine pituitary extract (0.4%), gentamicin (50 μg ml^{-1}) and amphotericin B (50 ng ml^{-1}). Human dermal fibroblasts derived from normal foreskin (Cascade Biologics) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, gentamicin (50 μg ml^{-1}) and amphotericin B (50 ng ml^{-1}). All experiments were carried out on cells at the third passage. In some experiments, keratinocytes were cultured until the fully confluent condition. After the removal of the medium, keratinocytes were exposed to air for 15 minutes with a lid (culture dish) or 30 minutes in the capped flask and incubated further 2 days with the medium containing 2 mM $CaCl_2$.

Skin samples

Biopsy samples were obtained at the Tokyo Medical University Hospital under informed consent and were used for immunohistochemical and *in situ* hybridization analyses. The study was approved by the Committee of Ethical Matters at the Tokyo Medical University School of Medicine and by the Shiseido Committee on Human Ethics. The study was conducted according to the Declaration of Helsinki Principles.

RT–PCR amplification and cDNA library screening

The following degenerate oligonucleotide primer pair, which corresponds to consensus sequences of human trypsinogens, was designed: sense primer 5′-TTCTGIGGIGGCTCCCTCAT-3′, antisense primer 5′-AGGCITGTTCITCTGGGCAC-3′. Total RNA (500 ng) isolated from cultured human keratinocytes was reverse transcribed using random hexamer and Superscript II RNase H-reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and then subjected to PCR amplification using Taq DNA polymerase (Takara, Kyoto, Japan) and the above primers. Forty cycles of amplification were performed at 94 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute. The PCR products of the expected size (540 bp) were gel-purified and subcloned into the pCR II vector (Invitrogen, San Diego, CA). The inserted PCR products were confirmed by a combination of restriction enzyme mapping and DNA sequencing. The sequences of all clones tested were identical with the sequences of PRSS3, and one of the clones (Tryp 1) was used as a probe for screening of a human keratinocyte cDNA library in the bacteriopharge λgt11 vector (Clontech, Mountain View, CA). DIG-labeled cDNA probes were prepared by PCR amplification of the insert using the primers and DIG-dUTP. Screening

was performed under high-stringency conditions and the obtained cDNA inserts of plaque-purified isolates were subcloned into pBluescript II KS + (Stratagene, La Jolla, CA) for DNA sequencing.

mRNA detection by RT–PCR

Total RNA from various tissues and cells (500 ng in each case) was reverse transcribed and then subjected to PCR amplification using specific forward primers for each type of trypsinogen; for detection of trypsinogens 1–3, degenerate forward primer 5′-ATCCA(C/T)TCCTGATCCTT(A/G)CC-3′, for trypsinogen 4 specific forward primer 5′-TTCTGGGTGGACGCACTTGG-3′, for trypsinogen 5 specific forward primer 5′-GTTCCGACTCGCATGGGACC-3′ and the common reverse primer for the trypsinogen family 5′-GGTAATCTTTCCAGGGTAGG-3′ were used. For enteropeptidase detection, the forward primer 5′-agctcaagacatcaccccaa-3′ and the reverse primer 5′-aaaccttgagaccctggcat-3′ were used. The glyceraldehyde-3-phosphate dehydrogenase gene was also amplified using the forward primer 5′-CCTGCTTCACCACCTTCT-3′ and the reverse primer 5′-CCCATCACCATCTTCCAG-3′, as a control for the amount of RNA loaded. Thirty cycles of amplification for trypsinogens and enteropeptidase, and 20 cycles for glyceraldehyde-3-phosphate dehydrogenase were performed at 94 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute. The PCR products were electrophoresed through 2% agarose gel and visualized by ethidium bromide staining and southern hybridization. PCR amplification of each RNA sample without reverse transcription was performed to verify the absence of genomic DNA contamination in each sample. Each product was digested with restriction enzyme to confirm its identity.

Southern hybridization

For Southern blot analysis of trypsinogens and enteropeptidase RT–PCR products, DIG-labeled cDNA probes were prepared using each PCR product as a template. After PCR and agarose gel electrophoresis, gel was stained with ethidium bromide, denatured in 0.5 N NaOH containing 1.5 M NaCl and transferred onto a nylon membrane. After fixation of DNA by UV cross-linking, hybridization was carried out at 65 °C overnight. The membrane was incubated with anti-DIG alkaline phosphatase conjugate and visualized with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate-*p*-toluidine salt (Roche Diagnostics, Indianapolis, IN).

Northern hybridization

Total RNA samples (1 μg) from cultured keratinocytes were subjected to electrophoresis in formaldehyde buffer and transferred onto a nylon membrane. After fixation of RNA by UV cross-linking, hybridization was carried out at 50 °C overnight with DIG-labeled cDNA probe synthesized from Tryp 1 plasmid by PCR. The membrane was incubated with anti-DIG horseradish peroxidase conjugate (Roche Diagnostics) and visualized with enhanced chemiluminescence plus reagents (GE Healthcare, Little Chalfont, UK).

In situ hybridization

In situ hybridization was performed on paraffin-embedded human skin sections. Human scalp skin sections were obtained with informed consent from patients who have undergone plastic surgery. DIG-labeled antisense and sense cRNA probes were synthesized from linearized Tryp 1 plasmid by *in vitro* transcription using a DIG RNA Labeling Kit (SP6/T7) (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's manual. Similarly, a cRNA probe for enteropeptidase was prepared from the PCR product as described above. The hybridization buffer consisted of 50% formamide, 5 × SSC, 5 × Denhardt's solution and the DIG-labeled probe (100 ng ml^{-1}), and hybridization was allowed to proceed at 55 °C for 16 hours. After posthybridization washing, the tissue sections were incubated with anti-DIG alkaline phosphatase conjugate (Boehringer Mannheim) and visualized with Vector Red (Vector Laboratories, Burlingame, CA) according to the manufacturer's manual.

Immunohistochemistry and TUNEL

Biopsy specimens (abdominal skins) were fixed with 4% paraformaldehyde. Anti-human cationic trypsin (rabbit IgG) (Athens Research & Technology; 1:500 dilution), anti-enteropeptidase IgG (mouse monoclonal) (R&D Systems, Minneapolis, MN; 1:200 dilution) and rabbit anti-ECS (rabbit IgG) (ECS, DDDDK-I) (Bethyl Laboratories, Montgomery, TX; 1:500) were used for immunostaining. Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR) was used for the fluorescence detection. Nuclei were visualized with 4′,6-diamino-2-phenylindole. The TUNEL reaction was performed using a fluorescein *in situ* cell death detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Western blot analysis

Keratinocyte extracts were separated with a 5–20% gradient gel by SDS-PAGE. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Human adult normal tissue proteins blot I was purchased from Alpha Diagnostic International (San Antonio, TX), in which tissue extracts from brain, colon, heart, kidney, liver, lung, pancreas and spleen were blotted on to a nitrocellulose membrane. Membranes were incubated with anti-human cationic trypsin antibody. Peroxidase-labeled anti-rabbit IgG (Sigma, St Louis, MO) was used as a secondary antibody, and immunoreactive proteins were visualized by chemiluminescence using enhanced chemiluminescence-plus (GE Healthcare).

Preparation of skin equivalent models

Skin equivalent models were prepared according to the method of Tsunenaga *et al.* (1998) and Amano *et al.* (2001). Briefly human keratinocytes were seeded on the surface of type I collagen gel contracted by human skin fibroblasts. After 2 days of culture, cornification was induced by lifting the gel to an air–liquid interface and the culture was continued up to 7 days until the well-differentiated cornified layer was formed. In another series of experiments, skin equivalent was fully immersed in the medium and continued to culture up to 7 days without air exposure. The medium used was a 1:1 mixture of keratinocyte grown medium and Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 1.8 mM $CaCl_2$ and 250 μM 2-*O*-α-D-glucopyranosyl-L-ascorbic acid.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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